REPRODUCTION AND LARVAL DEVELOPMENT OF ACMAEA TESTUDINALIS (MÜLLER)[1]

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Although the biology and anatomy of *Acmaea testudinalis* have been studied (Willcox, 1900a, 1905, 1906) its larval development has never been described. Isolated stages of this limpet have been studied by Morse (1910), Thompson (1912), Thorson (1946), and Raven (1958). Boutan (1898, 1899), with *Acmaea virginea*, and Thorson (1935), with *Acmaea rubella*, have been the only workers to present any details of larval development in this genus. This report describes and illustrates the developmental stages of *A. testudinalis*.

The tortoise-shell limpet is a gastropod of the order Diotocardia, superfamily Patellacea and family Acmaeidae (Fretter and Graham, 1962). It is found on rocks and shells just below the low tide line on the coasts of islands and continents bordering the North Atlantic and also around the Arctic Circle to the Bering Sea (Grant, 1937).

Great variation in reproductive methods exists in the genus *Acmaea*. *A. testudinalis* is unisexual and lays its eggs in a thin mucous sheet. *A. virginea*, also unisexual, lays its eggs singly (Boutan, 1898). *A. fragilis*, a protandric hermaphrodite for a short time, also lays its eggs singly (Willcox, 1898, 1900b). *A. rubella* is a viviparous hermaphrodite (Thorson, 1935). Fertilization is external in *A. testudinalis* and *A. virginea*, though Willcox (1900b) mentioned that the renal papilla of *A. fragilis* might act as an intromittant organ.

An exhaustive study of stimuli affecting reproductive cycles and the relation of these cycles to geographic location and habitat of 21 species and subspecies of *Acmaea* on the Pacific Coast was made recently by Fritchman (1961a, 1961b, 1961c, 1962). He found that air and water temperatures, geographic range, phase of the moon and tidal cycles all could influence gonad development and spawning. For instance, *A. insessa* spawned at any provocation; *A. fenestrata cribraria* spawned three times a year; *A. persona* spawned once a year from March to April. *Acmaea scutum*, the Pacific limpet so closely related to *A. testudinalis*, has several partial spawnings a year and at no time is the gonad indeterminant. Fritchman (1961b) feels that these spawnings may have some relation to the full moon and its tides.

Breeding in the British limpet, *Patella vulgata*, was studied by Orton, Southward and Dodd (1956). Both *P. vulgata* and *P. coerulea* are protandric hermaphrodites (Fretter and Graham, 1962) but *P. depressa* is unisexual (Orton and Southward, 1961). All three lay their eggs singly.

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Successful fertilization and rearing of larvae has been a problem confronting many workers who have investigated limpet development, as well as that of other organisms. Patten (1885) and later LoBianco in 1889 (Dodd, 1957) were successful in obtaining artificial fertilization with *Patella coerulea*. Unfortunately, Patten (1886), in studying the embryology of this species, was largely working with abnormal larvae (Dodd, 1957). However, Patten remained the primary source of information on limpet development until Smith (1935) published his work on development of *Patella vulgata*. Smith was able to raise a few to metamorphosis. Dodd (1951, 1957) has been successful in rearing large numbers of *P. vulgata* larvae through metamorphosis. Boutan (1899) raised larvae of *A. virginea* in tanks with sand-filtered running sea water, using algae, which came through the system, as food. Dodd's (1957) rearing method emphasized the importance of mechanical stirring of rearing jars from fertilization until the larvae began to settle.

Materials and Methods

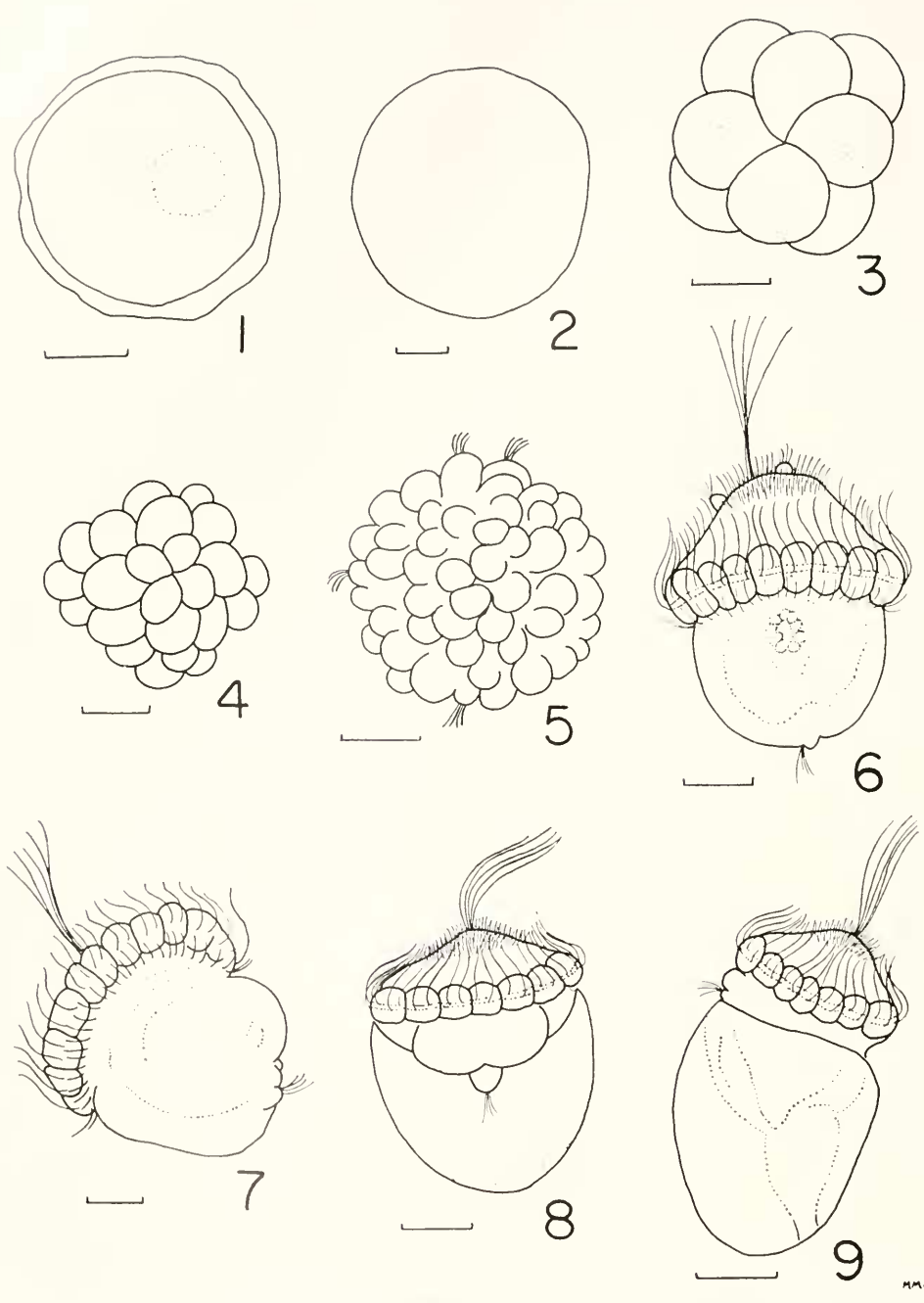
Adult limpets were collected at Noank, Connecticut, in June, July and December of 1962 and June of 1963, and at Watch Hill, Rhode Island, in May, July and August of 1962 and February, April and June of 1963. No extensive plankton samples were taken. Adults were kept in aquaria with running sea water in the Marine Research Laboratory at Noank and in the aquatic room of the Life Science building at Storrs. Microscopic algae on small rocks were provided for food.

Method of obtaining gametes

Ripe individuals were selected by observing the sole of the limpet's foot. Ripe males have a creamy streak down the underside of the foot, while females show a reddish area. This can best be seen through the glass side of an aquarium.

Two to four males and five to eight females were placed in acid-washed finger bowls (one for males and one for females) and brought out of the aquatic room where the water was $12.1 \pm 0.5°$ C., into room temperature and allowed to warm slowly. Spawning would occur when water reached 19.5° to 22.5° C. Limpets also responded to gentle agitation of the water or small squirts of water into the nuchal cavity at those temperatures. By having males and females releasing gametes in separate bowls, polyspermy can be prevented.

A tremendous amount of sperm is released, and is emitted for a longer period than the females' spawn. Each sperm is about 0.054 mm. long. Sperm are extremely active and remain viable for at least three hours. Eggs of *Acmaea testudinalis* are small (0.14 mm. in diameter) and tan to brick red in color. An egg taken from the ovary (Fig. 1) possesses an outer membrane which appears to swell on contact with water but later gradually disappears (Fig. 2). Unfertilized eggs fixed immediately after spawning show the eggs held in a sheet at regular intervals by their gelatinous coverings. Not all eggs are attached; some become free upon contact with the water. Therefore, it would appear that the thin mucous sheet is not formed by the sole of the foot, as Willcox (1905) suggested, but by agglutination of the gelatinous membranes about each egg.



A camera lucida attached to a Wild M20 phase contrast microscope with a 20× Fluotar objective and 10× ocular were used for drawings. Figures 14 and 18 were sketched without the camera lucida because the larvae were too active at the time to use this attachment. For clarity, the number of cilia shown is less than in the living specimen. Each scale line in the figures equals 0.05 mm. except in Figure 18 where it equals 0.1 mm.

When the animal is releasing gametes, the shell is lifted high off the substrate and tilted toward the posterior end. In the male, sperm is squirted from the renal papilla; in the female, eggs seem to be ejected from the nuchal cavity. Usually eggs remain unfertilized or cleave abnormally if gonads are removed from the animals, as Dodd (1957) did with *Patella*. Best results were obtained when the limpets were allowed to spawn naturally in separate bowls.

Method for rearing larvae

Adults were taken out of spawning bowls and the eggs then poured through cheesecloth to remove any debris or fecal matter. The eggs were separated into four or five dishes, depending upon the number released. Larvae developed better in numbers (at least 400 eggs per dish) than with too few per container. Two to four drops of sperm suspension were placed in each egg dish and stirred. The rearing temperature was 12.1 ±0.5° C.

Trochophores developed within 10 to 13 hours. Since they swam at the surface of the water, the top portion of the water could be poured into clean bowls and aerated filtered sea water added. This eliminated uncleaved and abnormal eggs which remained on the bottom of the dish.

Water to be used was kept in a plastic jug and aerated continuously with an air hose inserted through a hole in the cap of the jug. It was found harmful to larvae to aerate rearing bowls. Water was changed every other day by pouring larvae and water through plankton netting with small enough mesh to prevent loss of larvae. In this case, #12 plankton netting, with a mesh opening of 110 microns, was used. Larvae were rinsed quickly into a clean bowl with clean water. Each fingerbowl was filled with about 200 ml. of water. This manner of water-changing was carried out until the larvae began to settle. At that time, water was pipetted off and clean aerated water added to the same dish. If the ciliate population had increased, the healthy larvae were removed with a pipette to clean dishes.

Feeding began at about 60 hours. A few drops of a culture of the diatom, *Phaeodactylum* sp., were added every other day until the larvae began to creep, at which time a bottom food was required. This was provided by placing a piece of *Ulva* (*Cytosiphon* was also recommended by Dodd, 1957) in an acid-washed finger bowl with aerated filtered sea water. Within a few days, spores were deposited on the bottom and the *Ulva* removed. Other algae such as diatoms may also be deposited there. Creeping larvae were transferred to the prepared dish and each individual placed in a cleared area. Larvae could then obtain their food by scraping

FIGURE 1. Egg taken directly from ovary; note sheath.

FIGURE 2. Fertilized egg; note absence of sheath.

FIGURE 3. Eight-celled stage; two hours 50 minutes after fertilization.

FIGURE 4. Multicelled stage; note micromeres; view of animal pole; four hours 40 minutes after fertilization.

FIGURE 5. Cilia forming; nine and one-half hours after fertilization.

FIGURE 6. Ventral view of trochophore with stomodaeum; 25 hours after fertilization.

FIGURE 7. Ventro-lateral view of late trochophore; 30½ hours after fertilization.

FIGURE 8. Ventral view of early veliger; note position of foot and telotroch; 36 hours after fertilization.

FIGURE 9. Lateral view of veliger; note position of telotroch; 47 hours after fertilization.

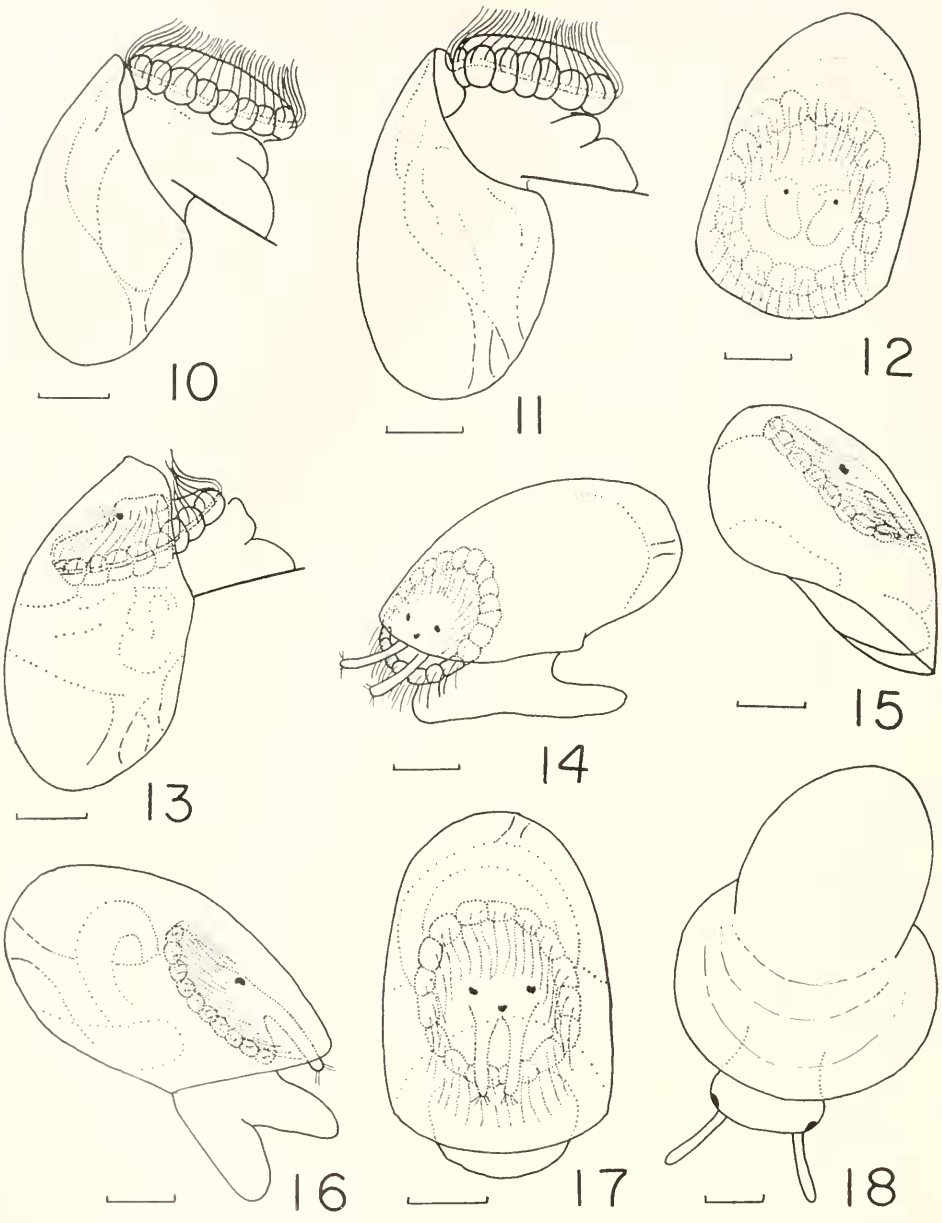


FIGURE 10. Lateral view of veliger after torsion; note position of muscle and foot; 62½ hours after fertilization.

FIGURE 11. Lateral view; note presence of second retractor muscle; 65 hours after fertilization.

FIGURE 12. Dorsal view of larva withdrawn into shell; note eyespots and tentacle rudiments; 85½ hours after fertilization.

FIGURE 13. Lateral view; 97½ hours after fertilization.

up algal spores and any diatoms present (Dodd, 1957). Settled larvae were moved to fresh algae with clean water every 7 to 10 days. Water was changed every other day by carefully pipetting or siphoning off old water and adding new. By this process, one larva was raised to metamorphosis (Fig. 18) but died at 46 days owing to a cracked shell, sustained when an attempt was made to clean some algal growth from it. Three others were also raised to metamorphosis. They had lost the velum but no peristome had begun to develop.

Observations

Reproduction

The release of gametes in *A. testudinalis* at Noank begins in late May and early June and continues until mid- or late-July, depending upon water temperature. In 1962, water temperature at Noank was about 17.5° C. in May, and about 21.0° C. in July. Willcox (1905) found limpets spawning as late as the first week in September at Eastport, Maine, and in August at Boston. She felt that spawning ended before water reached its maximum temperature.

Sexes cannot be determined externally from about September to November. Though the normal spawning period for this limpet is during the summer, three pairs spawned in the laboratory January 28, 1963, at about 17.0° C., and normal larvae developed. However, these particular limpets had been collected in December, 1962, and the warmer temperature of the aquatic room may have stimulated gonad activity. Thus, it may be possible to obtain ripe gametes before the normal breeding season. Female gonads appear to mature sooner than male; of 49 limpets collected February 19, 1963, at Watch Hill, 10 were female and 39 were indeterminant. It appears, therefore, that *A. testudinalis* spawns once a year during the summer months, with spawning triggered by warmer temperatures.

In the Noank laboratory, different paired individuals (one male and one female per bowl) spawned at about 19.7° C. in mid- and late-June and at about 21.5° C. in July. Spawning did not occur below 17.0° C. or above 22.5° C., except in one female that spawned at 24.0° C. on July 3, 1963.

On the average, males tended to release gametes at a slightly lower temperature than females. As July approached, with water temperatures of 20.0 to 22.0° C., the time gap between male and female spawning was narrowed. In July, with temperatures of about 22.0° C., gametes were released at almost the same time and temperature.

Development of eggs and larvae

The unfertilized egg (Fig. 1) soon lost its gelatinous sheath. Only after this sheath disappeared and the eggs became spherical and opaque could fertilization occur. No fertilization membrane was observed in living or stained zygotes. Two

Figure 14. Lateral view of creeping larva; note absence of operculum; 161½ hours after fertilization.

Figure 15. Lateral view of larva withdrawn into shell; 186½ hours after fertilization.

Figure 16. Lateral view of veliger partially retracted; 219½ hours after fertilization.

Figure 17. Dorsal view of retracted veliger; note size of eyes and tentacles; 235 hours after fertilization.

Figure 18. Dorsal view of 42-day-old larva.

polar bodies were visible in stained material 20 minutes after fertilization and in the two- and four-celled stages. Cleavage is spiral, complete, and unequal after four cells. Two cells formed one hour after fertilization, four cells at one hour 30 minutes, eight cells with micromeres (Fig. 3) at two hours 30 minutes and 12 cells at three hours. By four hours and 40 minutes after fertilization, the micromeres had become noticeably smaller (Fig. 4).

Short tufts of cilia were evident on certain cells at 9½ hours (Fig. 5). At 13 hours, with development of an apical tuft and prototrochal cilia, the trochophore began to swim. At 25 hours (Fig. 6) a ventral circular group of cells denoting the stomodaeum, a posterior tuft of cilia forming the telotroch, and two cilia-tufted apical cells flanking the apical tuft could be seen. Neither apical tuft nor apical cells perform a locomotor function but appear to have some sensory role. Further growth of prototrochal cilia enabled the phototactic larvae to swim more actively. Changes to the veliger stage were seen at 30 hours (Fig. 7), with proliferation of the shell gland on the dorsal side and ventral flexion of the telotroch toward the foot rudiment.

The shell, with its granulated surface, grew rapidly, encapsulating the larvae by 32½ hours. At that stage, the shorter posterior cilia of the prototroch (now velum) were no longer visible. With the aid of the circular velum (Fig. 8) the larvae rotated through the water with sudden short bursts of speed. At 47 hours the first retractor muscle was visible through the shell (Fig. 9). By 52 hours the telotroch had disappeared. The successive positions of the telotroch during ventral flexion may be followed in Figures 7, 8 and 9. Though less phototactic, the veliger still swam actively and began to feed.

Torsion occurred at 61 hours. At 62½ hours the apical tuft had disappeared and an operculum was evident (Fig. 10). It may be noted in Figure 9 that the retractor muscle and foot were on opposite sides of the shell and that after torsion (Fig. 10) they were on the same side. Partial retraction of the larva into the larval shell was possible. With formation of a second retractor muscle, seen at 65 hours (Fig. 11), complete retraction could occur (Figs. 12 and 15). The foot had developed into a creeping organ, and at 85 hours after fertilization larvae were both crawling and swimming. By that time, eyespots had formed and tentacle rudiments were evident (Fig. 12). During the course of development the eyespots increased in size and the tentacles became longer and tipped with short cilia (Fig. 17). At 100 hours the intestine was well formed and the larvae swam less and crept about more. The foot was provided with numerous tiny cilia which are not shown in the figures. In crawling, the larvae appeared to have difficulty balancing the larval shell.

Little outward change occurred in stages shown in Figures 14 through 17. But at 17 days the very beginning of the peristome was present, the velum and operculum had disappeared, and the head began to look quite "limpet-like." Figure 18 shows a beginning peristome which eventually encircles the base of the larval shell. Tentacles and eyes were most like the adult and a rudimentary radula was present.

A. testudinalis larvae are planktonic for approximately four days, about 19 hours of which are spent as trochophores and nearly 56 hours as veligers. Metamorphosis appears to begin at about 15 days. Complete development from egg to newly metamorphosed adult probably occurs in six weeks.

Discussion

Comparison of reproduction and larval development among seven species of prosobranchs

Reproduction and development in *Acmaea testudinalis* are contrasted here with four other diotocardians (*Haliotis tuberculata, Patella vulgata, Patina pellucida* and *Acmaea virginea*) and two monotocardians (*Crepidula fornicata* and *Littorina littorea*). In general, all of the diotocardians listed have external fertilization, planktonic eggs, free-swimming trochophores, veliger larvae and a brief pelagic life (Lebour, 1937; Fretter and Graham, 1962). The two monotocardians have internal fertilization, egg capsules (planktonic in *L. littorea*, attached in *C. fornicata*), free-swimming veligers and an extended pelagic life (Conklin, 1897; Lebour, 1937; Thorson, 1946; Fretter and Graham, 1962). All the diotocardians and monotocardians listed are unisexual except *Patella vulgata* and *Crepidula fornicata*, which are protandric hermaphrodites (Fretter and Graham, 1962). All spawn some time during the summer, except *P. vulgata* which is a winter breeder (Crofts, 1937; Lebour, 1937; Fretter and Graham, 1962).

Of these diotocardians, *A. virginea* has the smallest egg (120 μ diameter without the gelatinous sheath) (Fretter and Graham, 1962) and *Patina pellucida* the largest (320 μ even without the sheath) (Lebour, 1937). All eggs are spawned singly in this group (Lebour, 1937; Fretter and Graham, 1962) except those of *A. testudinalis* which are laid in a mucous sheet formed by agglutination of the gelatinous sheaths about each egg.

The trochophore stage of the diotocardians is planktonic but in the monotocardians this stage develops within the egg (Fretter and Graham, 1962). All diotocardian trochophores here have a long apical tuft except *Haliotis*, which has none (Crofts, 1937), and all have a monotrochal prototroch except *Patella* in which there are two rows of cells (Fretter and Graham, 1962). No telotroch is present in *Haliotis* (Crofts, 1937). One tuft of cilia forms this structure in *Patella*, probably *Patina* (Lebour, 1937; Fretter and Graham, 1962), and *A. testudinalis;* three tufts compose the telotroch of *A. virginea* (Boutan, 1899). All have short cilia covering the apical end except *Haliotis* (Fetter and Graham, 1962) and all are top-shaped except the trochophore of *Patina* which is pear-shaped (Lebour, 1937). Of the first four listed, *Patina* has the longest trochophore (200 μ) (Lebour, 1937) and *Haliotis* the shortest (130 μ) (Crofts, 1937).

Diotocardian veligers develop a small circular velum, while that of the monotocardians becomes large and bilobed (Fretter and Graham, 1962). The larval shell in diotocardians is capsule-shaped with no coiling, except possibly in *Patella* (Fretter and Graham, 1962) and in *Haliotis* where it is initially a cap but later acquires a one and one-half whorl spiral (Lebour, 1937). Both monotocardian larval shells are definitely spiral (Fretter and Graham, 1962). From the literature (Boutan, 1899; Dodd, 1957), veligers of *P. vulgata, A. virginea* and *A. testudi-* in *Haliotis, Patella* and *Patina* (Raven, 1958), but is a quick process in *A. virginea* (two to three minutes) (Boutan, 1899) and *A. testudinalis* (less than one hour). With *Littorina* and *Crepidula*, torsion is a gradual process occurring within the egg (Fretter and Graham, 1962).

Summary

1. At Noank, Connecticut, *Acmaea testudinalis* spawns from May to July.

2. Males tend to release gametes before females in June and early July, and thus in nature probably act as a stimulus to the females. However, in mid-July, males and females spawned at about the same time and temperature in the laboratory.

3. When laid, eggs of *A. testudinalis* appear to be in a thin mucous sheet formed by agglutination of the gelatinous sheath about each egg. This sheath swells on contact with water and soon disappears, leaving the egg free and ready for fertilization.

4. The photopositive trochophore larvae form 10 to 13 hours after fertilization and remain in this stage for about 19 hours.

5. Veligers develop 31 to 36 hours after fertilization and remain in that stage for about 56 hours. They become increasingly photonegative.

6. Complete development from egg to newly formed adult takes about six weeks.

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